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Quantitative analysis of adhesive resin in the hybrid layer using Raman spectroscopy

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Abstract

The objective was to determine absolute molar concentration of adhesive resin components in the hybrid layer by establishing methods based on Raman spectroscopy fundamentals. The hybrid layer was treated as a three-component system consisting of collagen and an adhesive resin containing two monomers. Adhesive standard specimens and Raman peak area ratios obtained with a 785 nm excitation wavelength were used to construct separate calibration curves for comonomer relative molar concentration and Bis-GMA absolute molar concentration. Since collagen and water had no measurable peaks in the fingerprint region, a dilution coefficient K_j was defined to describe their impact on Raman peak area and to calculate HEMA absolute molar concentration. Methodology was validated using an analogous system containing acetone/ethanol/water. The absolute molar concentration of Bis-GMA and HEMA decreased 87% and 83%, respectively, from the top quarter to the middle of the hybrid layer. Additionally, less Bis-GMA penetrated the hybrid layer than HEMA, as indicated by the ~20% decrease in comonomer molar concentration ratio between the adhesive resin layer and the top half of the hybrid layer. Lack of complete monomer infiltration will further challenge dentin-adhesive bond longevity.

Keywords

dentin; adhesive resin; Raman spectroscopy; hybrid layer; molar concentration

1. Introduction

In bonded restorations, failure mechanisms involving the adhesive resin-dentin interface, so-called ‘hybrid layer,’¹ are reported to involve both the demineralized dentin matrix and adhesive resin.² Few studies have attempted to measure the degree of adhesive resin infiltration into the hybrid layer. Most studies evaluating resin-dentin bonding efficiency are based on morphological^{3–5} and mechanical testing.^{6,7} Of those studies measuring adhesive resin infiltration into the demineralized dentin matrix,^{8–10} no study to date has reported absolute molar concentrations of resin components. Qualitative studies, reporting relative monomer concentration, consistently reveal the important finding of decreasing and non-uniform resin infiltration from the adhesive layer toward the base of the hybrid layer.^{11–13} However, absolute molar concentration measurements will provide a more thorough understanding of current adhesive performance and elucidate directions to be taken for future improvement.

Raman spectroscopy reflects the rotational and vibrational transitions of molecules. This technique is based on light scattering instead of absorption or transmission and has several distinct advantages for study of the resin-dentin bond: it is noninvasive, nondestructive, and water-insensitive.¹⁴ In addition, Raman has excellent spatial resolution (as low as 1

micrometer), making it one of the best techniques to acquire chemical information in the 4-10 micrometer-thick hybrid layer of etch-and-rinse systems.¹⁵ Currently, Raman spectroscopy has been used in dental research mainly to determine conversion of polymer systems and, to a lesser extent, detect infiltration of adhesive resin into the hybrid layer.¹⁵⁻¹⁹

When simplified to its most basic composition, the hybrid layer can be thought to consist of three components: comonomers of a binary adhesive system (e.g., Bis-GMA/HEMA or Bis-GMA/TEGDMA) and collagen of the demineralized dentin matrix. Ideally, the biological (collagen) and synthetic (adhesive resin) polymers within the hybrid layer should be quantified. Collagen density may not be constant throughout the hybrid layer; therefore, relative molar concentration of adhesive components may not accurately describe the complexity of the hybrid layer. Similarly, it will be beneficial to describe the adhesive infiltration in the hybrid layer using a method based on absolute molar concentration.

The objective of this study is to determine the absolute molar concentration of adhesive resin in the hybrid layer by establishing and validating methods based on the fundamentals of Raman spectroscopy. The hypothesis was tested that the absolute molar concentrations of Bis-GMA and HEMA polymers are not uniform across the hybrid layer.

2. Materials and Methods

2.1 Materials

Three sets of materials were used in this study: 1) an experimental adhesive system containing the comonomers 2-hydroxyethyl methacrylate (HEMA) and 2,2-bis(4-(2-hydroxy-3-methacryloxypropoxy)phenyl)propane (Bis-GMA) with the two-component photoinitiator system camphorquinone (CQ) and ethyl 4-dimethylaminobenzoate (EDMAB); 2) a commercial adhesive system for dentin bonding (Adper Scotchbond MultiPurpose, lot# 9KU, 3M ESPE, St. Paul, MN, USA); and 3) acetone (reagent grade, Fischer Scientific, Pittsburgh, PA, USA), ethanol (190 proof, Fischer Scientific), and deionized water for methodological verification. These chemicals were used as purchased with the exception that the primer in the commercial adhesive system was substituted with a 35:65 wt % ratio of HEMA:acetone for experimental simplification and comparative purposes to previous and ongoing studies.²⁰⁻²¹ The resin-dentin bonded sample preparation was fully described in a prior study.²²

2.2 Methods

2.2.1 Relationship between Raman intensity and molar concentration—

According to Raman theory, the radiant power of Raman scattering (Φ_R) is directly proportional to the Raman cross-section, $\sigma(\nu_{ex})$:²³

$$\Phi_R \propto \sigma(\nu_{ex}) \nu_{ex}^4 E_0 n_i e^{-E_i/kT} \quad (1)$$

where ν_{ex} is excitation wavelength, E_0 is source irradiance, n_i is the number density in state i , and the exponential term is the Boltzmann factor for state i , which is a function of temperature (T). In accordance with Eqn 1, studies have shown that, although the value of the Raman cross-section varied with different wavelengths, it has a specific value at each wavelength.²⁴ In addition, the Raman cross-section dispersions of condensed and vapor phases were found to be identical for benzene, cyclohexane, and acetonitrile.²⁴ In this present study, only one Raman instrument and one three-step etch-and-rinse adhesive

system were used; therefore, the Raman cross-section, source irradiance, excitation laser wavelength, and temperature were assumed constant. Since only one microscope objective was used for collecting spectra, it was also assumed that the Raman sampling volume (V_s) was constant. By multiplying the right-hand side by V_s/V_s , Eqn 1 was rewritten as:

$$\Phi_R \propto K \left(\frac{n_i}{V_s} \right) \quad (2)$$

where $\frac{n_i}{V_s}$ is molar concentration and K is the product of V_s , $\sigma(\nu_{ex})$, ν_{ex} , E_0 , and the Boltzmann factor.

As mentioned above, the hybrid layer can be treated as a three-component system consisting of collagen and an adhesive resin containing two monomers. The contribution of initiators in the adhesive system was neglected because their initial concentrations make up less than 2 wt % of the adhesive formulation. The irradiant source in this experimental setup, a 785 nm laser, does not induce a detectable Raman peak for collagen in the fingerprint region (200-2000 cm^{-1}) (see Figure 1). Therefore, to simplify the three-component system, collagen was treated as a Raman inactive material, and its main effect on the collected Raman spectra of adhesive within the hybrid layer, as well as any water present, was to dilute the molar concentration of adhesive components accordingly.

2.2.2 Preparation and characterization of standards for the adhesive system—

A series of adhesive standard specimens was prepared with increasing concentration of Bis-GMA (0 to 98 wt %). The initiator (CQ) and co-initiator (EDMAB) were held constant at 1 wt % each, with HEMA making up the balance for each adhesive standard specimen. Three specimens at each concentration were photopolymerized at room temperature by illumination for 60 s in aluminum sample pans (approximately 50 μL) with a plasma-arc light curing unit (Virtuoso Xenon PAC, Den-Mat Corp., Santa Maria, CA, USA) positioned 1 cm from the specimens. The irradiance at the light guide tip was 1400 mW/cm^2 as measured by a radiometer with a wavelength range of 400-500 nm (L.E.D. Radiometer, Demetron, Danbury, CT, USA).

Raman spectra of specimens were then collected using an optical microscope (DMLP, Leica Microsystems GmbH, Wetzlar, DEU) attached to a modular research Raman spectrograph (HoloLab5000R, Kaiser Optical Systems, Inc., Ann Arbor, MI, USA) using a 785 nm laser at 8 mW to induce the Raman scattering effect. The spectral coverage of this model ranges from 100 to 3450 cm^{-1} with an average spectral resolution of 5 cm^{-1} . Spectra were obtained under a 100 $\times$ objective with an exposure time of 60 s. Instrumental calibration was performed before each experiment using a calibration accessory (HCA-RXN, Kaiser Optical Systems, Inc.) to manufacturer specifications, and all measurements were performed with auto-dark subtraction and cosmic-ray correction.

After acquiring spectra, adhesive standard molar concentrations were calculated by determining the mass of each photopolymerized specimen to within 0.1 mg (Analytical Balance, AB104-S, Mettler-Toledo, Inc., Columbus, OH, USA) and the volume gravimetrically by displacement methods. The moles (N_i) of Bis-GMA and HEMA in each specimen were determined using Eqn 3:

$$N_i = \frac{m \times w_i}{M_i} \quad (3)$$

where m is the mass of the specimen, w_i is the mass fraction of component i , and M_i is the molecular weight of i . The corresponding molar concentrations, c_i , of Bis-GMA and HEMA for each specimen were then determined using Eqn 4:

$$c_i = \frac{N_i}{V} \quad (4)$$

where V is the volume of each specimen.

Raman spectral profiles were used to determine the relative Bis-GMA and HEMA molar concentration of the polymerized adhesive specimens. As shown in Figure 1, two characteristic peaks were selected: for Bis-GMA alone (1610 cm^{-1}) and HEMA and Bis-GMA together (605 cm^{-1}). In this binary comonomer system, each peak area ratio of 1610 cm^{-1} over 605 cm^{-1} corresponds to a pair of Bis-GMA and HEMA molar concentrations. The following equation expresses the relationship between the relative Bis-GMA molar concentration and the Raman spectral data:

$$\text{Bis - GMA molar concentration} \propto \frac{A_{1610}}{A_{605}} = \frac{[\text{Bis - GMA}]}{[\text{Bis - GMA}] + [\text{HEMA}]} \quad (5)$$

where the A_{1610} (aromatic ring) peak area is directly related to the Bis-GMA molar concentration and the A_{605} (unchanging carbonyl stretch) peak area is directly related to the combined concentration of HEMA and Bis-GMA.²⁵

A calibration curve establishing the relative concentration relationship between these monomers when copolymerized was developed by mixing increasing amounts of Bis-GMA in HEMA in calibration standards, keeping CQ and EDMAB concentrations at 1 wt % each (see Figure 2). The molar concentration of Bis-GMA and HEMA was calculated using Eqns 3 and 4. Five Raman spectra of each photopolymerized adhesive standard were obtained, and averaged integrated peak areas were used to calculate the Raman peak area ratio in Eqn 5. Linear regression of the data resulted in an excellent fit ($R^2 = 0.9888$ for HEMA and $R^2 = 0.9846$ for Bis-GMA) between the two parameters, enabling a direct correlation between the Raman peak area ratio and the molar concentration of Bis-GMA and HEMA in the adhesive specimens.

In the binary system of the adhesive layer, Figure 2 can be directly used to determine the absolute molar concentration of Bis-GMA and HEMA. However, in the three-component system of the hybrid layer where collagen is treated as Raman inactive, Figure 2 can only be used to determine the relative molar concentrations of Bis-GMA and HEMA, since the calibration curve does not take into account the dilution effect of the collagen and other “Raman inactive” constituents within the hybrid layer.

A calibration curve establishing the absolute molar concentration of Bis-GMA was developed using the same set of adhesive standard specimens and Raman spectra (see Figure 3). The molar concentration of Bis-GMA was calculated using Eqns 3 and 4. Five Raman spectra of each photopolymerized adhesive standard were obtained, and the averaged

integrated peak area at 1610 cm^{-1} for each of the adhesive standards (A_{1610}) was normalized by the averaged integrated peak area of the 0 wt % HEMA standard specimens ($A_{1610}^{98\text{wt}\% \text{Bis-GMA}}$). Linear regression of the data resulted in an excellent fit ($R^2 = 0.9928$) between the two parameters, enabling a direct correlation between the Raman peak area and the molar concentration of Bis-GMA in the adhesive specimens ($c_{\text{Bis-GMA}}^{\text{abs}}$), as predicted in Eqn 2:

$$R_{1610} = \frac{A_{1610}}{A_{1610}^{98\text{wt}\% \text{Bis-GMA}}} = 0.4347 c_{\text{Bis-GMA}}^{\text{abs}} + 0.2196 \quad (6)$$

2.2.3 Determination of absolute molar concentration in the three-component systems—After the calibration curves were developed (Figures 2 and 3 for the adhesive system), the following procedure was performed to determine the absolute molar concentration in the three-component resin-dentin system, consisting of HEMA, Bis-GMA, and the “Raman inactive” collagen. Nine Raman spectra were taken across the intertubular hybrid layer at seven positions of the resin-dentin bond (see Figure 4: pure adhesive layer, top of the hybrid layer, top quarter of the hybrid layer, middle of the hybrid layer, bottom quarter of the hybrid layer, bottom of the hybrid layer, and dentin), as described in an earlier study.²²

The absolute molar concentration of Bis-GMA in the hybrid layer was determined by normalizing averaged peak areas at 1610 cm^{-1} acquired at point j within the hybrid layer (A_{1610}^j) to the averaged peak area at 1610 cm^{-1} collected from the adhesive resin (AR) layer of the same resin-dentin sample (A_{1610}^{AR}). This normalized Raman peak area was used with the linear best fit from Figure 3 to determine the absolute molar concentration of Bis-GMA at point j in the hybrid layer ($c_{\text{Bis-GMA}, j}^{\text{abs}}$):

$$\frac{R_{1610}^j}{R_{1610}^{\text{AR}}} = \frac{A_{1610}^j}{A_{1610}^{\text{AR}}} = \frac{0.4347 c_{\text{bis-GMA}, j}^{\text{abs}} + 0.2196}{0.4347 c_{\text{bis-GMA}, \text{AR}}^{\text{abs}} + 0.2196} \quad (7)$$

where $c_{\text{Bis-GMA}, \text{AR}}^{\text{abs}}$ is the absolute molar concentration of Bis-GMA in the adhesive resin layer. The difference between the relative and absolute molar concentration of Bis-GMA within the hybrid layer stems from the “dilution effect” caused by the presence of collagen and any other “Raman inactive” constituents. To determine the absolute molar concentration of HEMA, this dilution effect must be quantified. Therefore, a dilution coefficient at each point j in the hybrid layer, K_j , was calculated from the Bis-GMA concentration data:

$$K_j = \frac{c_{\text{Bis-GMA}, j}^{\text{abs}}}{c_{\text{Bis-GMA}, j}^{\text{rel}}} \quad (8)$$

where $c_{\text{Bis-GMA}, j}^{\text{abs}}$ is the absolute molar concentration of Bis-GMA at point j obtained from Figure 3 and $c_{\text{Bis-GMA}, j}^{\text{rel}}$ is the relative molar concentration of Bis-GMA at point j obtained from Figure 2.

Once K_j was calculated at each location within the hybrid layer, the corresponding absolute molar concentration of HEMA, $c_{HEMA, j}^{abs}$, was calculated as follows:

$$c_{HEMA, j}^{abs} = K_j * c_{HEMA, j}^{rel} \quad (9)$$

where $c_{HEMA, j}^{rel}$ is the relative molar concentration of HEMA at point j obtained from Figure 2. This methodology was verified using an analogous ethanol/acetone/water system (see Appendix).

2.2.4 Statistical methods—The absolute molar concentrations for Bis-GMA and HEMA obtained from the dentin-adhesive specimen at the adhesive resin layer, top quarter of the hybrid layer, and middle of the hybrid layer were submitted to ANOVA with Dunnett's multiple comparison *post hoc* test with a significance level set at $\alpha = 0.05$ using a statistical software package (Prism v. 4.00, GraphPad Software, Inc., San Diego, CA, USA).

3. Results

Molar concentrations were calculated from the averaged Raman peak areas at 605 and 1610 cm^{-1} , the Bis-GMA/HEMA calibration curves (Figures 2 and 3), and K_j calculated from the Bis-GMA concentration data. In resin-dentin specimens, the adhesive peak intensity significantly decreased relative to baseline for depths greater than the middle of the hybrid layer. Thus, reliable concentration data could be reported only for the first four regions from the pure adhesive resin layer to the middle of the hybrid layer. The molar concentrations for both Bis-GMA and HEMA were significantly lower in the top quarter and middle of the hybrid layer ($p < 0.0001$) relative to their concentrations in the pure adhesive resin layer: 0.55 and 0.07 mol/L, respectively, for Bis-GMA and 1.81 and 0.30 mol/L, respectively, for HEMA, compared to 1.53 and 3.23 mol/L for the overlying adhesive layer (Table 1).

4. Discussion

The absolute molar concentration of adhesive components (Bis-GMA and HEMA) significantly decreased across the hybrid layer; therefore, the tested hypothesis of non-uniform monomer distribution throughout the hybrid layer was accepted. Bis-GMA and HEMA absolute molar concentrations decreased 87% and 83%, respectively, from the top quarter of the hybrid layer position to the middle of the hybrid layer. In addition, less Bis-GMA penetrated the hybrid layer than HEMA, as indicated by the ~20% drop in the comonomer molar concentration ratio between the pure adhesive layer and the middle of the hybrid layer (see Table 1). For locations deeper in the hybrid layer, reliable data could not be obtained due to low signal-to-noise ratio. However, it is reasonable to expect that the molar concentrations of Bis-GMA and HEMA will be even lower than that reported at the middle of the hybrid layer. The results clearly demonstrate that neither HEMA nor the cross-linking dimethacrylate Bis-GMA monomer fully infiltrated the demineralized dentin matrix. If a region of the hybrid layer is primarily infiltrated with the mono-functional HEMA monomer, only linear polymer chains will be present, in lieu of a cross-linked polymer network. Such a situation will further challenge dentin-adhesive bond integrity and increase susceptibility to plasticization and hydrolysis. Although conversion studies described earlier²²⁻²⁵ have presented a similar and detailed discussion concerning the effect of a HEMA-rich area in the bottom of the hybrid layer, this study provides direct measurement of Bis-GMA/HEMA monomer distribution and, through the measurement of absolute concentrations, portrays a bleaker picture. Since dentin consists of 45-50% inorganic apatite

crystals, ~30% organic matrix, and 20-25% water by volume, the expected concentration of monomer throughout the hybrid layer should be roughly 50% of that measured in the adhesive layer.²⁶ Therefore, Bis-GMA and HEMA molar concentrations should have measured around 0.765 and 1.615 mol/L, respectively, throughout the hybrid layer, which is not the case as shown in this study. An incompletely infiltrated hybrid layer may explain the large strains measured by microscopic moiré interferometry across the compressive loaded resin-dentin interface²⁷ and limited clinical durability.²⁸

Data collected from the top of the hybrid layer was not included in the statistical testing due to concerns of possible “contamination” of adhesive layer signals in data collected from the top of the hybrid layer. This concern was validated by absolute molar concentrations at the top of the hybrid layer for Bis-GMA and HEMA of 1.17 ± 0.13 and 3.02 ± 0.56 mol/L, respectively, that are far beyond that expected, as mentioned above, by potential volume occupancy within the hybrid layer alone.

One feature of this study was to simplify the three-component hybrid layer system to a pseudo two-component system. Generally, for a three-component system, a series of calibration curves must be developed to determine molar concentration. However, the number of standards needed to develop calibration curves would be exceedingly large. In addition, there is no unity relationship among these three components present in the hybrid layer. In other words, there were three independent concentration variables instead of two found in a typical three-component system. Therefore, even though a three-component calibration determination is theoretically possible, it is not achievable within the hybrid layer. The present method eliminates the necessity of three-component calibration curves by exploiting collagen's lack of Raman signal when using a 785 nm laser. With no spectral interference from collagen, this method also did not require, unlike other reported methods, 29 complicated peak subtraction or fitting.

To determine accurately the absolute monomer molar concentration, the contribution of the demineralized dentin matrix (primarily Type I collagen) in the hybrid layer must be addressed. In this method, the dilution coefficient K_j was used to take into account the effect of any “Raman inactive” components, mainly collagen, at different depths in the hybrid layer. To the authors' best knowledge, this is the first report of the absolute molar concentration of adhesive comonomers across the hybrid layer. Although the value of K_j decreases from the top of the hybrid layer to the middle of the hybrid layer, this may not necessarily mean that the density of collagen increases towards the bottom of the hybrid layer. On the contrary, less effective penetration of comonomers into the hybrid layer accounts for a preponderance of the declining K_j . The declining K_j through the hybrid layer represents an increasing free volume taken up by residual water and dentinal fluids.

Another benefit of this method is that it paves the way to study more complicated dental adhesive bonding systems. Most current dental adhesive systems contain more than two monomers, making it very difficult to measure quantitatively the distribution of the adhesive components within the hybrid layer. However, it is anticipated that this method can be applied to similar or even more complicated systems in the future. Also, although there are some morphological and bond strength studies that have demonstrated changes in the hybrid layer after long-term media storage,^{30,31} there are no reports detailing adhesive concentration changes over time. If the poly(HEMA) components of the hybrid layer are lost faster than the poly(Bis-GMA) components, the analytical approach described in the current report should be able to confirm that prediction.

In this study, the linear relationship between characteristic peak area and the absolute molar concentration was proposed based on two assumptions: first, the Raman cross-section,

source irradiance, excitation laser wavelength, and temperature can be assumed constant to simplify Eqn 1; second, the Raman sampling volume (V_s) can be assumed constant if only one microscope objective was used throughout the study. The validity of this new method and its two assumptions was verified using an analogous system containing acetone, ethanol, and water, where the Raman spectrum of water behaved similarly to collagen in the hybrid layer. The correlation between true molar concentrations and calculated molar concentrations of the standards in the external prediction set was excellent (Figures A4 and A5). Therefore, the assumptions used to derive the molar concentration equations (Eqns 8 and 9) are reasonable, and the determination of absolute molar concentrations of Bis-GMA and HEMA in the dentin-adhesive bond is an acceptable extension of this method. Nevertheless, additional verification experiments using other analogous systems or cross-checking studies using a corroborating technique, such as fluorescence-tagged monomers, may need to be performed to verify this method so that further benefits of this method could be realized. Since the Raman cross-section of species may change with phase or be affected by environmental changes, studies of Raman spectra from both liquid monomer systems and polymerized adhesive dissolved in monomers may lead to a better understanding regarding the assumptions made in this study.

In the present study, unlike what was seen in the analogous system (Figure A3), the y-intercept was non-zero for the Bis-GMA absolute molar concentration calibration curve (Figure 3). This may be due to the presence of the co-initiator (EDMAB), which also contains an aromatic ring that adds to the 1610 cm^{-1} peak area. Since the initial concentration of EDMAB is small, the contribution was not obvious when the Bis-GMA concentration was large; however, when the Bis-GMA concentration was very low, the contribution from the co-initiator may have become apparent. The accuracy of the calculation may vary at different locations. For example, in the adhesive layer, the molar concentration of Bis-GMA was determined as 1.53 mol/L , which fell between the values of two calibration standards ($c_{\text{Bis-GMA}} = 1.444\text{ mol/L}$ (corresponding to 58.6 wt %) and $c_{\text{Bis-GMA}} = 1.612\text{ mol/L}$ (corresponding to 70.3 wt %)). This is in agreement with the adhesive resin composition provided by the manufacturer (i.e., 60-70 wt % Bis-GMA).²⁵ However, less than 10% Bis-GMA was observed at the middle of the hybrid layer. In this position, the 1610 cm^{-1} peak intensity is very low, and uncertainty exists as to the contributions from Bis-GMA or EDMAB. Therefore, the acquisition of absolute adhesive molar concentrations and data analysis at the middle of the hybrid layer (and deeper) must be very carefully interpreted and deserves further study.

5. Conclusion

In this study, a method was developed to determine the absolute molar concentrations of monomers in the hybrid layer of dentin-resin bonds. The validity of this method was verified using an analogous system containing acetone/ethanol/water, for which the calculated and true molar concentration of acetone and ethanol agreed well. The absolute molar concentrations of Bis-GMA and HEMA in top half of the hybrid layer were determined and clearly demonstrated a significant decrease in Bis-GMA and HEMA from the overlying adhesive resin layer to the middle of the hybrid layer.

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8. Appendix: Methodological Validation

8.1 Method

Since the preparation of homogenous and reliable adhesive resin-collagen standards is extremely complex, the new methodology described in Sections 2.2.2 and 2.2.3 was validated using an acetone/ethanol/water system. This system was chosen for several reasons: these three components can be homogeneously mixed; water, analogous to collagen in the Bis-GMA/HEMA/collagen system, is a poor Raman scatterer; and acetone and ethanol possess distinct spectra with well-defined characteristic peaks.

8.1.1 Preparation and characterization of standards for the analogous system

A series of standards was prepared with increasing concentration of ethanol (0-100 wt %) with acetone making up the balance for each acetone/ethanol standard. Using these standards, a relative molar concentration calibration curve for the analogous system (ethanol/acetone) and an absolute molar concentration calibration curve for ethanol were constructed. In addition, an external prediction set, consisting of seven acetone/ethanol/water solutions (see Table A1), was prepared to check the validity of the proposed method. To achieve homogeneity, all standard solutions were shaken manually for at least 1 min. For each standard, its acetone, ethanol, and/or water molar concentrations were calculated using Eqns 3 and 4 as described in Section 2.2.2.

For Raman spectral acquisition, each standard was transferred to a clean, capped quartz vial to prevent evaporation of the volatile components. Raman spectra were acquired using a holographic fiber-coupled stretch probehead (Mark II, Kaiser Optical Systems, Inc.) attached to the modular research Raman spectrograph. The characteristic Raman peaks of the three pure components are shown in Figure A1. Five spectra for each standard were collected using an exposure time of 100 ms and laser power of 250 mW. Wavelength and intensity calibrations were performed using the calibration accessory to manufacturer specifications before each experiment.

Raman spectral profiles were used to determine the relative ethanol and acetone molar concentrations of the analogous system specimens. In this binary system, the following equation expresses the relationship between the relative acetone molar concentrations and the Raman spectral data:

$$\text{acetone molar concentration} \propto \frac{I_{780}}{(I_{780} + I_{880})} = \frac{[\text{acetone}]}{[\text{acetone}] + [\text{ethanol}]} \quad (\text{A1})$$

where the I_{780} (CCC stretching³²) peak intensity is directly related to the acetone molar concentration and the I_{880} (CCO symmetric stretching^{33,34}) peak intensity is directly related to the ethanol molar concentration.

A calibration curve establishing the relative concentration relationship between these solvents was developed by mixing increasing amounts of ethanol in acetone (see Figure A2). Five Raman spectra of each analogous system standard were obtained, and averaged peak intensities were used to calculate the Raman peak intensity ratio in Eqn A1 since the spectra were well-defined, characteristic peaks for acetone and ethanol were resolved, and all spectra were obtained the same day under similar conditions. Regression (second-order polynomial) of the data resulted in an excellent fit ($R^2 = 0.9979$ for both acetone and

ethanol) between the two parameters, enabling a direct correlation between the Raman peak intensity ratio and the molar concentrations of ethanol and acetone in the specimens.

A calibration curve establishing the absolute concentration of ethanol was developed using the same set of analogous system specimens and Raman spectra (see Figure A3). Five Raman spectra of each analogous system standard were obtained, and the averaged peak intensity at 880 cm^{-1} for each of the standards was normalized to that of the 100 wt % ethanol standard specimens. Linear regression of the data resulted in an excellent fit ($R^2 = 0.9994$) between the two parameters, enabling a direct correlation between the Raman peak intensity and the molar concentration of ethanol in the specimens, as predicted in Eqn 2.

8.1.2 Determination of absolute molar concentration in the analogous system

The procedure described in Section 2.2.3 was verified for the analogous system using the external prediction set listed in Table A1, the averaged Raman peak intensities at 780 and 880 cm^{-1} , the ethanol/acetone calibration curves (Figures A2 and A3), and K_j calculated from the ethanol concentration data. In this analogous system, ethanol (the analog for Bis-GMA) and acetone (the analog for HEMA) are Raman active, while water (corresponding to the collagen in the adhesive system) is “Raman inactive.” The relative and absolute molar concentrations of ethanol and acetone were calculated for each solution in the external prediction set and compared to the actual absolute molar concentration.

8.2 Results

The absolute molar concentrations of ethanol and acetone were calculated for each solution in the external prediction set and compared to the actual absolute molar concentrations (Figures A4 and A5, respectively). Linear regression of the data resulted in a one-to-one correlation between the calculated and measured concentrations ($R^2 = 1.00$ for ethanol and $R^2 = 0.9815$ for acetone), thus validating the data of the analogous Bis-GMA/HEMA/collagen system.

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Figure 1.

Characteristic peaks in Raman spectrum of HEMA and Bis-GMA: internal reference peak at 605 cm^{-1} , C=C double bond reactive peaks at 1410 and 1640 cm^{-1} , Bis-GMA benzene ring at 1610 cm^{-1} , and carbonyl peak at 1720 cm^{-1} . No characteristic peaks were identified in the Raman spectrum of the demineralized dentin. (Figure 2 in Ref 25)

Figure 2.

Molar concentration calibration curves for photopolymerized system (Bis-GMA and HEMA with CQ/EDMAB photoinitiator system) based on the Raman peak area ratio of the Bis-GMA aromatic ring peak at 1610 cm^{-1} and the methacrylate CCO peak at 605 cm^{-1} .

Figure 3. Absolute molar concentration calibration curve for Bis-GMA. The peak area of the Bis-GMA aromatic ring peak at 1610 cm⁻¹ was normalized by the standard with the highest Bis-GMA concentration (98 wt % Bis-GMA, 1 wt % CQ, 1 wt % EDMAB). Note: linear best fit is shown as dashed line.

Figure 4.

Transmission electron micrograph of the resin-dentin hybrid layer depicting where Raman spectral data were analyzed. Note the electron-lucent spaces around cross-sectioned collagen fibrils that contain adhesive resin. AR: adhesive resin, THL: top of hybrid layer, TQHL: top quarter of hybrid layer, MHL: middle of hybrid layer, BQHL: bottom quarter of hybrid layer, BHL: bottom of hybrid layer. Resin composite not shown. (Original magnification 16,000 ×) (Modified from Figure 2 in Ref 22)

Figure A1.

Raman spectrum of acetone, ethanol, and water: acetone characteristic peak at 780 cm⁻¹ (CCC stretching) and ethanol characteristic peak at 880 cm⁻¹ (CCO symmetric stretching). No characteristic peaks were identified in the Raman spectrum for water.

Figure A2.

Molar concentration calibration curve for homogeneous acetone/ethanol system based on the Raman peak intensity ratio of acetone peak intensity at 780 cm^{-1} and the sum of the ethanol peak intensity at 880 cm^{-1} and acetone peak intensity at 780 cm^{-1} .

Figure A3.

Absolute molar concentration calibration curve for ethanol. The peak intensity of the ethanol peak at 880 cm⁻¹ was normalized to the peak intensity of pure ethanol. Note: linear best fit is shown as dashed line.

Figure A4.

Comparison of the molar concentration of ethanol calculated from the calibration curves in Figures A2 and A3 and the true molar concentration of ethanol in the acetone/ethanol/water three-component system. Note: linear best fit is shown as dashed line.

Figure A5.

Comparison of the true molar concentration of acetone in the acetone/ethanol/water three-component system with the molar concentration of acetone calculated from the calibration curves in Figures A2 and A3 and the dilution coefficient K_j . Note: linear best fit is shown as dashed line.

Table 1

Absolute molar concentration of Bis-GMA and HEMA in the hybrid layer.

	Bis-GMA (mol/L $\pm$ SD)	HEMA (mol/L $\pm$ SD)	K _j	Bis-GMA:HEMA molar conc ratio	Total monomers
AR	1.53 $\pm$ 0.10 ^a	3.23 $\pm$ 0.43 ^a	1.00	0.47	4.76
THL	1.17 $\pm$ 0.13	3.02 $\pm$ 0.56	0.82	0.39	4.19
TQHL	0.55 $\pm$ 0.18 ^b	1.81 $\pm$ 0.54 ^b	0.43	0.30	2.36
MHL	0.07 $\pm$ 0.14 ^c	0.30 $\pm$ 0.54 ^c	0.06	0.23	0.37

¹ K_j = dilution coefficient; SD = standard deviation; AR = adhesive resin; THL = top of hybrid layer, TQHL = top quarter of hybrid layer, MHL = middle of hybrid layer.

² Within columns, groups with a different letter are significantly different.

³ THL position was not included in statistical analyses.

⁴ Molar concentrations of Bis-GMA and HEMA in adhesive layer corresponded to adhesive system containing 65 wt % Bis-GMA (falling between the adhesive standard data points cBis-GMA = 1.444 mol/L at 58.6 wt % and cBis-GMA = 1.612 mol/L at 70.3 wt %), which fell in the range of commercial adhesive composition (60-70 wt % Bis-GMA; 40-30 wt % HEMA).

Table A1

External prediction set for analogous system containing acetone, water, and ethanol.

Testing Standard	Acetone, mol/L	Ethanol, mol/L	Water, mol/L
1	13.60	0	0
2	3.92	7.05	16.67
3	2.49	8.49	17.82
4	1.77	10.46	14.37
5	7.48	5.01	8.74
6	3.03	2.03	36.56
7	0	17.13	0